Chapter 4

Effects of growth hormone-releasing peptides on the release of adenohypophyseal hormones in healthy dogs and in dogs with pituitary-dependent hyperadrenocorticism

S.F.M. Bhatti[1], S.P. De Vliegher[2], L.M.L. Van Ham[1], H.S. Kooistra[3]

Molecular and Cellular Endocrinology, 2002;197:97-103.

[1]Department of Small Animal Medicine and Clinical Biology,
[2]Department of Reproduction, Obstetrics, and Herd Health,
Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[3]Department of Clinical Sciences of Companion Animals,
Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Abstract

The aim of this study was to investigate the effects of ghrelin and growth hormone-releasing peptide-6 (GHRP-6) on the release of growth hormone (GH), adrenocorticotrophic hormone (ACTH), cortisol, thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and prolactin (PRL) in dogs with pituitary-dependent hyperadrenocorticism (PDH) and in healthy dogs of comparable age. In eight healthy dogs, the responses to ghrelin and GHRP-6 were compared to those of GH-releasing hormone (GHRH) and NaCl 0.9 % (control). In seven dogs with PDH, the effects of ghrelin and GHRP-6 were compared with their effects in healthy dogs.

In the healthy dogs, GHRH, GHRP-6, and ghrelin caused a significant rise in plasma GH concentrations. Administration of GHRH elicited significantly higher plasma GH concentrations than administration of ghrelin and GHRP-6. In the dogs with PDH, the GHRP-6-induced release of GH was significantly lower than in healthy dogs. Administration of ghrelin elicited a GH release that did not differ significantly between dogs with PDH and healthy dogs. Ghrelin and GHRP-6 did not cause a significant rise in plasma ACTH, cortisol, TSH, LH, and PRL concentrations in either the healthy dogs or the dogs with PDH.

It is concluded that in comparison with GHRH, GHRP-6 and ghrelin have a low GH-releasing potency in healthy dogs. In dogs with PDH, the GH release in response to GHRP-6 is impaired. Neither GHRP-6 nor ghrelin activates the pituitary-adrenocortical axis or stimulates TSH, LH, and PRL release in healthy elderly dogs and dogs with PDH.

Introduction

For many years it was thought that the pulsatile secretion of growth hormone (GH) by pituitary somatotrophs was controlled by the two antagonistic hypothalamic peptides GH-releasing hormone (GHRH) and somatostatin (SS) (Plotsky and Vale, 1985). However, GH release can also be elicited by synthetic GH secretagogues (GHSs) (Bowers et al., 1977, Momany et al., 1981). Synthetic GHSs exert their effect on GH release by acting through receptors different from those for GHRH (Casanueva and Dieguez, 1999). Growth hormone-releasing peptide-6 (GHRP-6) was the first powerful GHS used in humans and rats. Nowadays, GHRP-6 is the gold standard against which all so-called non-classic GHSs are compared (Korbonits and Grossman, 1995). After the introduction of GHRP-6, a new generation of peptidyl and non-peptidyl GHSs was developed (Casanueva and Dieguez, 1999). Interestingly, most of the GHSs were constructed well before the isolation of GHRH in 1982 (Guillemin et al., 1982, Rivier et al., 1982). In 1996, the GHS-receptor (GHS-R), a specific G-protein-coupled seven-transmembrane receptor, was identified by Pong et al. The GHS-R is present in various tissues (e.g. pituitary, hypothalamus, heart, lung, pancreas, intestine, adipose tissue). The demonstration of a hypothalamic and pituitary localization of the human GHS-R is consistent with its role in regulating GH release. The expression of this receptor in other central and peripheral regions may imply that it is involved in additional yet undefined physiological functions (Guan et al., 1997; Papotti et al., 2000; Kojima et al., 2001).

In 1999, Kojima et al. purified and characterized the endogenous ligand for the GHS-R from rat and human stomach and called it ‘ghrelin’. Ghrelin has since been identified in the fundus of the stomach of dogs and appears to be highly conserved (Tomasetto et al., 2001). Unlike the digestive enzymes, ghrelin is not secreted into the gastrointestinal tract but released into the bloodstream to act - among other possible functions - on the pituitary to release GH. Ghrelin is a peptide of 28 amino acids, in which the hydroxyl group of the serine 3 residue is esterified by *n*-octanoic acid. This octanoylation is essential for its GH-releasing activity (Kojima et al., 1999).

In healthy humans, the endocrine effects of GHSs are not specific for GH. Administration of synthetic GHSs also significantly increases the secretion of prolactin (PRL), adrenocorticotrophic hormone (ACTH), and cortisol (Massoud et al., 1996, Arvat et al., 2001). According to Kojima et al. (1999), ghrelin specifically stimulates GH release and does not affect the secretion of other adenohypophyseal hormones in rats. In contrast, Arvat et

al. (2001) demonstrated that intravenous administration of ghrelin, apart from stimulating GH secretion, also increases circulating PRL, ACTH, and cortisol levels in healthy humans.

Several pathological (e.g. obesity and chronic hypercortisolism) and non-pathological (e.g. ageing) states in humans are characterized by a reduction in pituitary GH secretion (Leal-Cerro et al., 1994). Chronic hypercortisolism is not only associated with reduced pituitary GH release (Hartog et al., 1964) but also with an impaired GH response to various stimuli (Casanueva, 1992). Even a combination of GHRH and GHRP-6, which is a very powerful GH-releasing stimulus, is unable to induce significant GH release in humans with Cushing's syndrome (Leal-Cerro et al., 1994). So far, there are no reports on the effects of ghrelin on GH release in patients with hyperadrenocorticism.

The aim of this study was to investigate the effects of ghrelin and GHRP-6 on the release of GH, ACTH, cortisol, thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and PRL in dogs with pituitary-dependent hyperadrenocorticism (PDH) and in healthy dogs of comparable age.

Materials and methods

Dogs

For the first study, eight healthy Beagle dogs (four males and four females) with ages ranging from 7 to 12 years (median age: 10 years) were used. In the second study, seven dogs of different breeds (seven males, two intact females and one spayed female), aged 9 to 13 years (median age: 10 years), with PDH were studied.

Diagnosis of hyperadrenocorticism was based upon elevated ($> 10 \times 10^{-6}$) corticoid/creatinine (C/C) ratios in two consecutive morning urine samples (Rijnberk et al., 1988). After collection of the second urine sample, the dogs received three oral doses of dexamethasone (0.1 mg/kg body weight) at 8-h intervals. Then a third urine sample was collected. If the C/C ratio in this third urine sample was less than 50 % of the mean of the first two samples, PDH was diagnosed (Galac et al., 1997). In one dog in which the urinary C/C ratio was suppressed less than 50 %, the diagnosis of PDH was confirmed by an elevated plasma ACTH concentration, bilaterally enlarged adrenal glands on ultrasonographic examination, and an abnormal pituitary gland on computed tomography (Voorhout, 1990, Voorhout et al., 1990).

Study design and blood sample collection

The first study was a randomized clinical trial in which the eight healthy Beagle dogs were randomly allocated to four groups. According to a Latin-square design, the dogs received an intravenous injection of either human GHRH (2 µg per kg body weight), GHRP-6 [(His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2)[3]] (2 µg per kg body weight), human ghrelin (2 µg per kg body weight) (Peninsula Laboratories Inc. Belmont, CA, USA), or 0.9 % NaCl (control). In order to avoid carry-over effects, washout periods of at least 4 days were included in the protocol.

In the second study, the seven dogs with PDH received an intravenous injection of either GHRP-6 (2 µg per kg body weight) or ghrelin (2 µg per kg body weight). One week later, the other compound was administered.

Blood samples for the determination of plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL were collected by jugular venipuncture at -15 and 0, 5, 10, 20, 30, and 45 min and immediately transferred to ice-chilled EDTA-coated tubes (GH, ACTH, cortisol, LH and PRL) or heparin-coated tubes (TSH). Samples were centrifuged at 4^{o} C for 10 min. Plasma was stored at -25^{o} C until assayed.

Hormone determination

The urinary corticoid concentrations were measured with a non-commercial radioimmunoassay (RIA). The urinary corticoid concentration was related to the urinary creatinine concentration and the corticoid/creatinine ratio was calculated (Stolp et al., 1983; Rijnberk et al., 1988).

Plasma GH concentrations were determined with a homologous RIA (Eigenmann and Eigenmann, 1981). The intra- and interassay coefficients of variation were 3.8 % and 7.2 % respectively, and the sensitivity of the assay was 0.3 µg/l. The degree of cross-reaction with canine PRL was 2 %.

Plasma ACTH concentrations were measured with an immunoradiometric assay (Nichols Institute, Wijchen, The Netherlands). The interassay coefficient of variation was 7.8 % and the sensitivity was 0.2 pmol/l.

Plasma cortisol concentrations were measured with a commercially available RIA (Diagnostic Products Corporation, Los Angeles, CA, USA), validated for the dog. The intra- and interassay coefficients of variation ranged from 3.0 % to 5.1 % and from 4.0 % to 6.4 %, respectively. The sensitivity of the assay was 5.5 nmol/l.

Plasma TSH concentrations were determined with a homologous solid-phase, two-site chemiluminescent enzyme immunometric assay (Immulite canine TSH, Diagnostic Products Corporation [DPC]) according to the instructions of the manufacturer. The intra-assay coefficients of variation were 5.0 %, 4.0 %, and 3.8 % at TSH levels of 0.20, 0.50, and 2.60 µg/l, respectively. The interassay coefficients of variation were 6.3 % and 8.2 % at TSH levels of 0.16 and 2.80 µg/l, respectively. The sensitivity of the assay was 0.03 µg/l. Cross-reactivity with FSH and LH was negligible.

Plasma LH concentrations were determined with a heterologous RIA as described previously by Nett et al. (1975). A rabbit antiserum raised against ovine LH (CSU-204; kindly supplied by G.D. Niswender, Colorado State University), radioiodinated NIAMDD-bLH-4, and canine pituitary standard LER 1985-1 (a gift from Dr. L.E. Reichert, Albany Medical College, NY) were used in this assay. The intra- and interassay coefficients of variation for values higher than 0.5 µg/l were 2.3 % and 10.5 % respectively. The sensitivity of the assay was 0.3 µg/l.

Plasma concentrations of PRL were determined with a previously validated heterologous RIA (Okkens et al., 1985). The intra-assay and interassay coefficients of variation were 3.5 % and 11.5 %, respectively. The sensitivity of the assay was 0.8 µg/l.

Statistical analysis

The plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL at -15 and 0 min were averaged per dog and used in all analyses as the basal plasma hormone concentration (basal).

Statistical analyses were performed using the MIXED procedure (SAS Version 8.02, SAS Institute Inc., Cary, NC, USA) with dog within group as the unit of analysis. Dog was considered a random effect. A first-order autoregressive covariance structure was used to model the autocorrelation in the repeated measures of the response variables. All data were log-transformed for further analysis to correct for non-normality.

For the first study, models were fitted for GH, ACTH, cortisol, TSH, LH, and PRL respectively, with compound (ghrelin, GHRP-6, GHRH, and 0.9 % NaCl), group of dogs (1 to 4), day of compound administration (day 1 to 4), and time (repeated measurement of hormones starting with the basal concentration), included as fixed effects. It was assumed there were no interactions between effects. For the second study, models were fitted separately for the responses of GH, ACTH, cortisol, TSH, LH, and PRL respectively, after ghrelin or GHRP-6 administration. Group (healthy dogs versus dogs with PDH), time

(repeated measurement of hormones starting with the basal concentration), and the interaction between group and time, allowing different slopes for both groups over time, were included as fixed effects. Values are expressed as mean ± SEM concentrations. Statistical significance was defined at $P \leq 0.05$.

Ethics of the study

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University.

Results

No side effects were observed during or after administration of the GH-releasing agents. In the healthy dogs, administration of ghrelin, GHRP-6, and GHRH resulted in a mean plasma GH concentration of 1.8 ± 0.5 µg/l, 2.7 ± 1.0 µg/l, and 5.5 ± 1.0 µg/l, respectively, which was significantly higher than that after administration of NaCl 0.9 % (0.9 ± 0.2 µg/l). GHRH administration caused a significantly higher mean plasma GH concentration (5.5 ± 1.0 µg/l) than administration of ghrelin (1.8 ± 0.5 µg/l) or GHRP-6 (2.7 ± 1.0 µg/l) (Table 1 and Figure 1).

Administration of the GH secretagogues did not cause stimulation of the pituitary-adrenocortical axis. Administration of ghrelin (25 ± 5 ng/l), GHRP-6 (30 ± 6 ng/l), and GHRH (21 ± 4 ng/l) resulted in mean plasma ACTH concentrations that did not differ significantly from those measured after administration of NaCl 0.9 % (25 ± 5 ng/l). Furthermore, administration of ghrelin (64 ± 19 nmol/l), GHRP-6 (80 ± 24 nmol/l), or GHRH (55 ± 8 nmol/l) resulted in mean plasma cortisol concentrations that were not significantly higher than those measured after administration of NaCl 0.9 % (73 ± 13 nmol/l) (Table 1 and Figure 1).

In the healthy dogs, the mean plasma TSH, LH, and PRL concentrations did not differ significantly when treated with the GH-releasing agents or NaCl 0.9 % (Table 1 and Figure 1).

Table 1. Mean (± SEM) plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL in eight healthy dogs treated with different GH secretagogues (GHSs) (ghrelin, GHRP-6, GHRH) and NaCl 0.9 % (control).

	Ghrelin	GHRP-6	GHRH	Control
GH (µg/l)	1.8 ± 0.5 **a,b**	2.7 ± 1.0 **a,c**	5.5 ± 1.0 **a,b,c**	0.9 ± 0.2
ACTH (ng/l)	25 ± 5	30 ± 6 **b**	21 ± 4 **b**	25 ± 5
Cortisol (nmol/l)	64 ± 19 **a**	80 ± 24 **b**	55 ± 8 **a,b**	73 ± 13
TSH (µg/l)	0.26 ± 0.07	0.24 ± 0.06	0.28 ± 0.09	0.23 ± 0.05
LH (µg/l)	3.8 ± 1.2	5.5 ± 1.5	3.5 ± 1.0	4.1 ± 1.6
PRL (µg/l)	3.4 ± 0.9	2.5 ± 0.5	2.8 ± 0.6	3.0 ± 0.4

a: significant difference in hormone concentrations between GHS and control group.

b, c: significant difference in hormone concentrations between indicated GHSs.

Administration of GHRP-6 to the healthy dogs elicited a mean plasma GH concentration of 2.7 ± 1.0 µg/l, which was significantly higher than the level in the dogs with PDH (0.8 ± 0.2 µg/l). Administration of ghrelin (1.8 ± 0.5 µg/l) led to a mean plasma GH concentration in the healthy dogs that did not differ significantly from that in the dogs with PDH (1.5 ± 0.7 µg/l). Neither GHRP-6 nor ghrelin caused a significant rise in plasma ACTH and cortisol concentrations in dogs with PDH. (Table 2, Figures 2 and 3). The mean plasma TSH, LH, and PRL concentrations in the healthy dogs after GHRP-6 or ghrelin administration did not differ significantly from that in the dogs with PDH and these hormone concentrations were not stimulated by the GHSs (Table 2, Figures 2 and 3).

Table 2. Mean (± SEM) plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL in seven dogs with pituitary-dependent hyperadrenocorticism (PDH) and eight healthy dogs treated with GHRP-6 and ghrelin.

	GHRP-6		Ghrelin	
	PDH	Healthy	PDH	Healthy
GH (µg/l)	0.8 ± 0.2 **a**	2.7 ± 1.0 **a**	1.5 ± 0.7	1.8 ± 0.5
ACTH (ng/l)	120 ± 30 **a**	30 ± 6 **a**	114 ± 24 **a**	25 ± 5 **a**
Cortisol (nmol/l)	246 ± 53 **a**	80 ± 24 **a**	174 ± 26 **a**	64 ± 19 **a**
TSH (µg/l)	0.21 ± 0.06	0.24 ± 0.06	0.24 ± 0.05	0.26 ± 0.07
LH (µg/l)	6.3 ± 2.6	5.5 ± 1.5	7.3 ± 2.7	3.8 ± 1.2
PRL (µg/l)	3.6 ± 0.9	2.5 ± 0.5	4.1 ± 1.3	3.4 ± 0.9

a: significant difference in hormone concentrations between dogs with PDH and healthy dogs

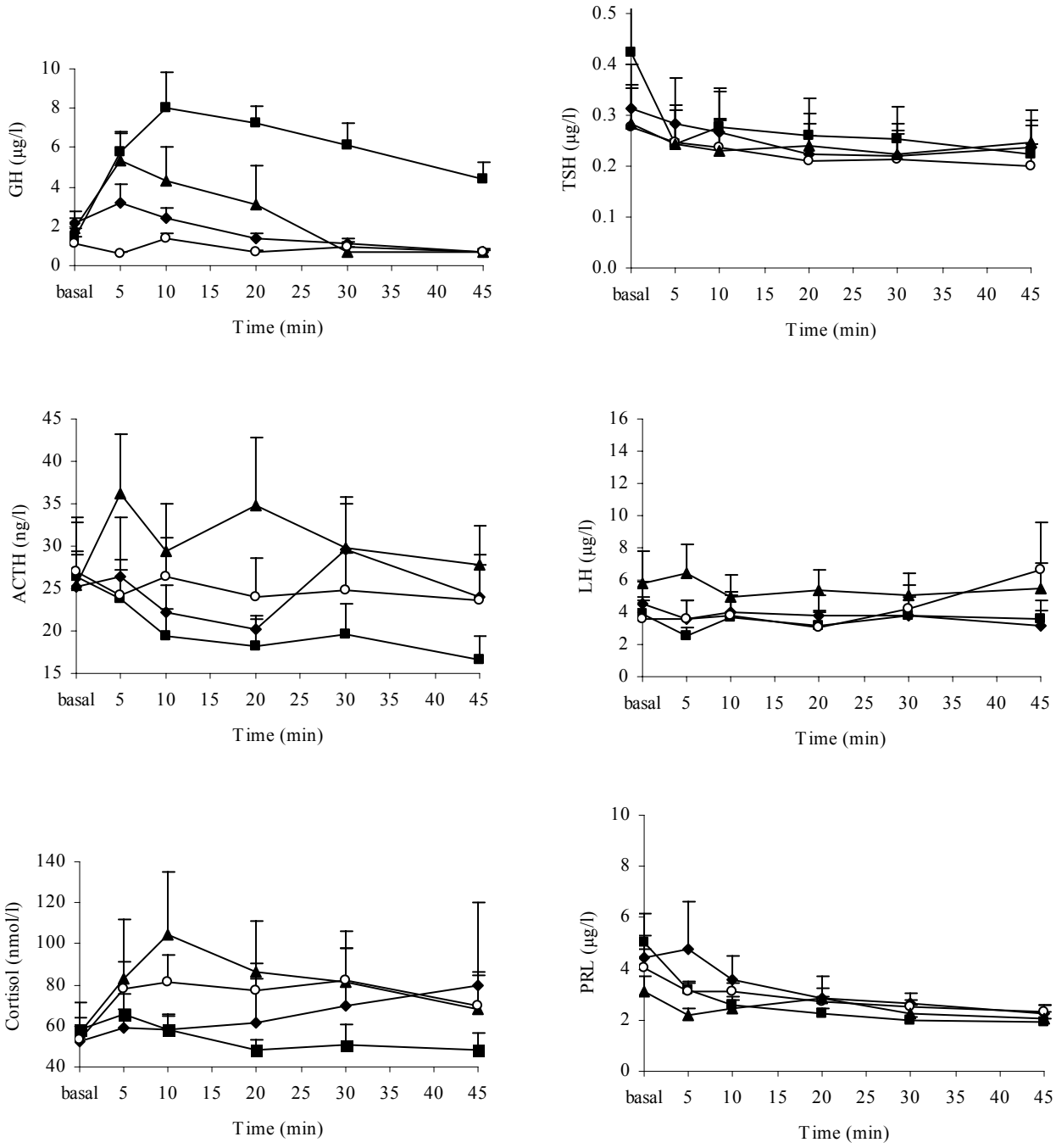

Figure 1. Mean (+ SEM) plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL after intravenous administration of ghrelin (♦), GHRP-6 (▲), GHRH (■), or NaCl 0.9 % (○) in eight healthy dogs.

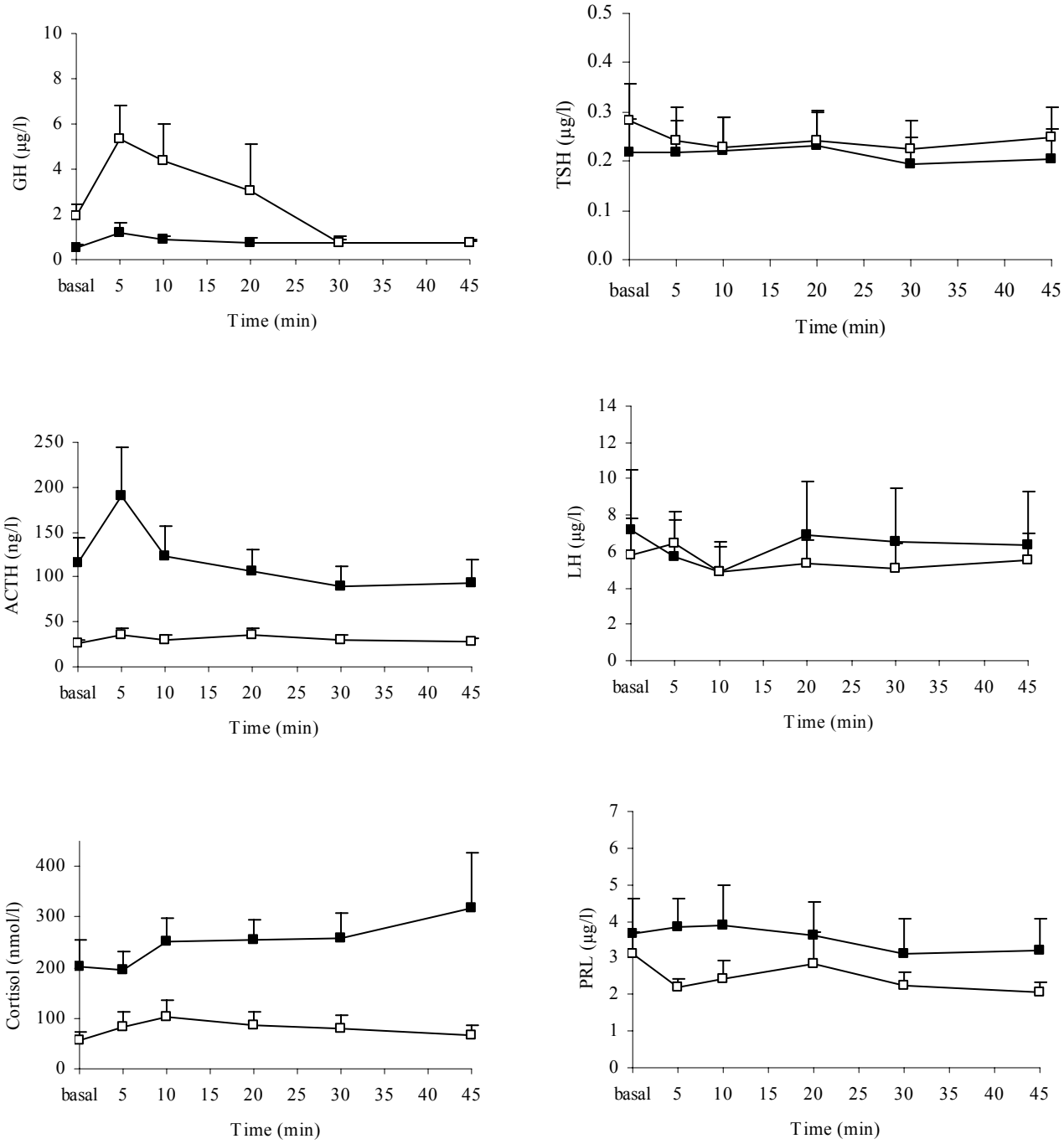

Figure 2. Mean (+ SEM) plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL after administration of GHRP-6 in eight healthy dogs (□) and in seven dogs with pituitary-dependent hyperadrenocorticism (PDH) (■).

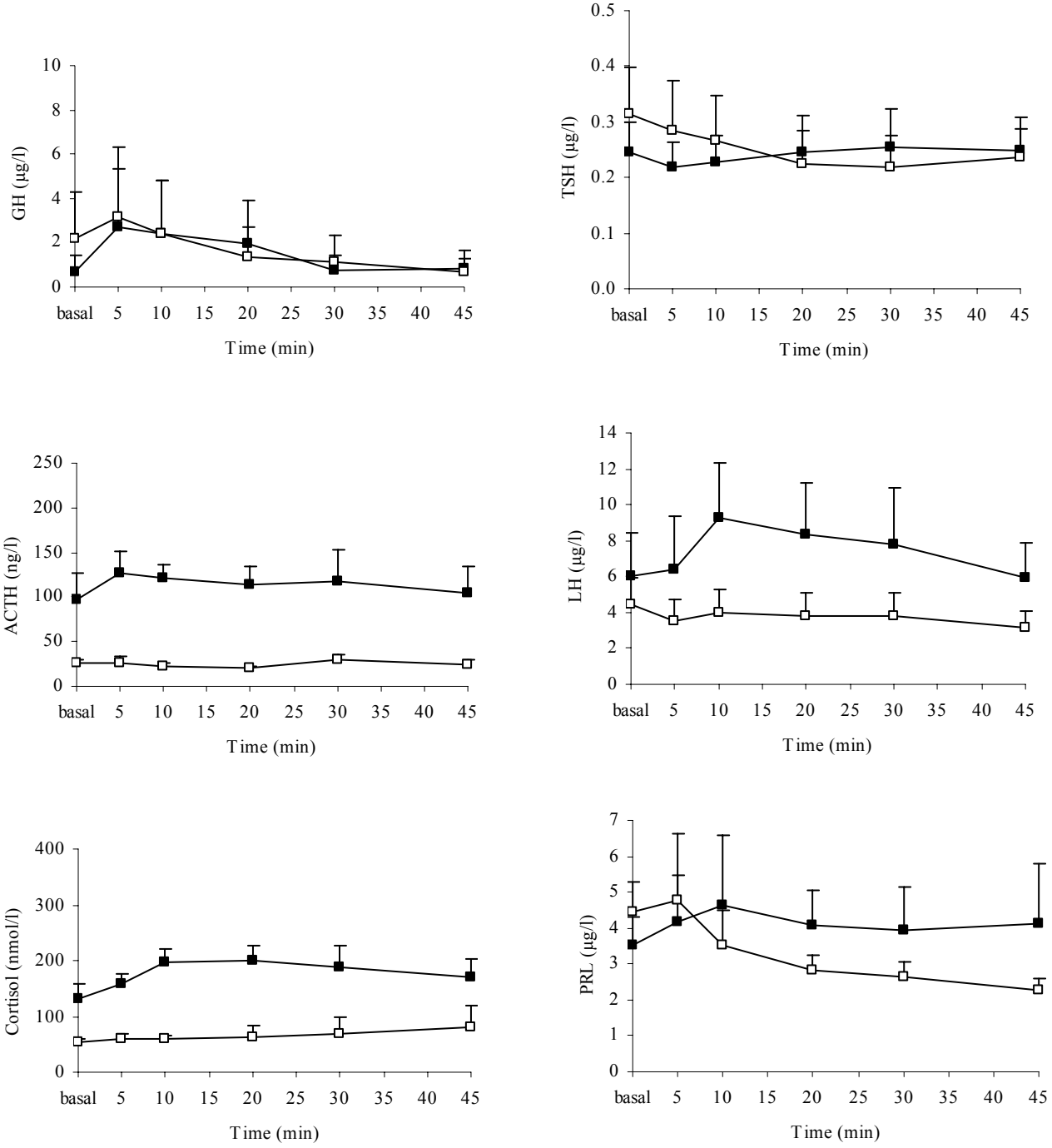

Figure 3. Mean (+ SEM) plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL after intravenous administration of ghrelin in eight healthy dogs (□) and in seven dogs with pituitary-dependent hyperadrenocorticism (PDH) (■).

Discussion

The results of this study demonstrate that both the natural and a synthetic ligand of the GHS-R (ghrelin and GHRP-6, respectively) stimulate GH release in healthy dogs. The results also underline the existence of intriguing species differences with regard to the GH-releasing potency of GHSs. In rats, the GH-releasing potency of ghrelin is similar to that of GHRH (Kojima et al., 1999), whereas in humans, ghrelin is a more potent stimulus of GH secretion than GHRH or the synthetic GHS hexarelin (Arvat et al., 2001). The results of the present study indicate that, compared with the GH-releasing effect of GHRH, ghrelin and GHRP-6 are not very potent stimulators of GH release in elderly dogs.

The GH response to stimulation with GHRH in the healthy dogs was considerably lower than that reported previously in healthy Beagle dogs (Meij et al., 1996). This may at least partly be due to the effects of ageing, because the healthy Beagle dogs in the study of Meij et al. (1996) had a median age of two years. It has been reported that GH secretion decreases in humans with increasing age (Finkelstein et al., 1972; Zadik et al., 1985; Wilshire et al., 1995) and in aged rats (Sonntag et al., 1980; De Gennaro Colonna et al., 1994). The decreased GH secretion in elderly humans has been ascribed to a blunted response of GH to GHRH (Shetty and Duthie, 1995) and feedback disruption of the regulatory GH-insulin-like growth factor-I system (Veldhuis, 1997). The decreased GH secretion in elderly rats is associated with a decreased pituitary GH content (Sonntag et al., 1980), reduced pituitary GH mRNA (Takahashi et al., 1992), and reduction of hypothalamic GHRH mRNA levels (De Gennaro Colonna et al., 1994). Also in the dog there are indications that ageing is associated with an impaired GH secretion. Cella et al. (1995) have demonstrated that the GH responsiveness to hexarelin, a synthetic GHS, decreases with ageing in the dog. This was previously shown for GH-releasing peptides in humans (Bowers et al., 1992) and rats (Walker et al., 1990). Data for aged dogs reinforce the idea that the pituitary mechanisms subserving GH secretion are, at least partially, preserved during ageing. Thus, the reduced GH secretion is mainly due to inadequate hypothalamic stimulation rather than a primary pituitary hypofunction (Franchimont et al., 1989; Walker et al., 1991; Cella et al., 1993). Because of the effects of ageing on GH secretion, in the present study dogs with PDH were compared with healthy dogs of comparable age.

Chronic hypercortisolism is associated with impaired somatic growth, reduced GH release (Hartog et al., 1964), and a blocked GH response to various GH stimuli (Casanueva, 1992). Even a combination of GHRH and GHRP-6, which is a very powerful GH-releasing

stimulus, is unable to induce significant GH release in humans with Cushing's syndrome (Leal-Cerro et al., 1994). The results of the present study demonstrate that administration of GHRP-6 also results in a blunted GH response in dogs with PDH. The GH response after administration of ghrelin to dogs with PDH and healthy dogs was low and not significantly different. The mechanism by which chronic glucocorticoid excess inhibits GH secretion is not yet fully understood. Several hypotheses have been proposed, the most important of which are GHRH hyposecretion, enhancement of hypothalamic SS release, or a combination of these (Leal-Cerro et al., 1998). In addition to the effect at the hypothalamic level, chronic glucocorticoid excess may also influence GH secretion by acting directly at the pituitary level (Leal-Cerro et al., 1994).

Polyphagia, polyuria, polydipsia, thin coat, alopecia, and muscular atrophy are the classical clinical manifestations of PDH in dogs (Rijnberk et al., 1968). Another cardinal physical feature is centripetal obesity with abdominal enlargement. As not only chronic hypercortisolism but also obesity is associated with an impaired GH response to GH-releasing stimuli (Bowers, 1993), it can be hypothesized that the suppressed GH release in Cushing's syndrome is related to obesity as well. Indeed, Leal-Cerro et al. (1998) demonstrated that in humans with Cushing's disease the hyporesponsiveness of somatotrophs to GHRH is improved after a short-term (3 days) hypocaloric diet. Indeed, GH secretion is increased after fasting in healthy humans (Ho et al., 1988) and healthy dogs (Arce et al., 1991), indicating that caloric restriction exerts an important stimulatory effect on GH secretion. This may be explained by two concurrently operating mechanisms, an increase in hypothalamic GHRH and a decrease in hypothalamic somatostatin, leading to increased plasma GH levels (Ho et al., 1988). However, in contrast to the situation of chronic hypercortisolism (Leal-Cerro et al., 1994), intravenous administration of the combination of GHRH and GHRP-6 resulted in an elevated GH response in obese humans (Bowers, 1993). This indicates that the impaired GH response in individuals with Cushing's syndrome cannot be explained solely by obesity.

Administration of ghrelin and GHRP-6 did not cause a stimulation of the pituitary-adrenocortical axis and did not stimulate TSH, LH, and PRL release in either the healthy dogs or the dogs with PDH. These results are in line with observations for anaesthetized rats reported by Kojima et al. (1999), who found that intravenous administration of ghrelin specifically stimulated GH release but did not affect other adenohypophyseal hormones. In contrast, Arvat et al. (2001) demonstrated that intravenous administration of ghrelin, apart from stimulating GH release, also increased circulating levels of PRL, ACTH, and cortisol in

healthy humans. So far, there is no explanation for this discrepancy other than species differences.

In conclusion, the results of this study demonstrate that, in comparison with GHRH, GHRP-6 and ghrelin have a low GH-releasing potency in healthy dogs. In dogs with PDH, the GH response to GHRP-6 is impaired. Neither GHRP-6 nor ghrelin activates the pituitary-adrenocortical axis or stimulates TSH, LH, and PRL release in healthy elderly dogs and dogs with PDH.

Acknowledgements

The statistical advice of Prof. Dr. M.-T. Ysebaert was highly appreciated. The authors are grateful for the technical assistance of Mr. H.G.H. van Engelen and Mrs. J. Wolfswinkel.

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